

miR-28-3p Is a Cellular Restriction Factor That Inhibits Human T Cell Leukemia Virus, Type 1 (HTLV-1) Replication and Virus Infection*

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Background: HTLV-1 has not been reported to be regulated by microRNA.

Results: We discovered that cellular miR-28-3p reduces virus replication and infection.

Conclusion: miR-28-3p may play an important role in limiting virus spreading.

Significance: miR-28-3p may represent a therapeutic target for HTLV-1-infected patients.

Human T cell leukemia virus, type 1 (HTLV-1) replication and spread are controlled by different viral and cellular factors. Although several anti-HIV cellular microRNAs have been described, such a regulation for HTLV-1 has not been reported. In this study, we found that miR-28-3p inhibits HTLV-1 virus expression and its replication by targeting a specific site within the genomic gag/pol viral mRNA. Because miR-28-3p is highly expressed in resting T cells, which are resistant to HTLV-1 infection, we investigated a potential protective role of miR-28-3p against *de novo* HTLV-1 infection. To this end, we developed a new sensitive and quantitative assay on the basis of the detection of products of reverse transcription. We demonstrate that miR-28-3p does not prevent virus receptor interaction or virus entry but, instead, induces a post-entry block at the reverse transcription level. In addition, we found that HTLV-1, subtype 1A isolates corresponding to the Japanese strain ATK-1 present a natural, single-nucleotide polymorphism within the miR-28-3p target site. As a result of this polymorphism, the ATK-1 virus sequence was not inhibited by miR-28. Interestingly, genetic studies on the transmission of the virus has shown that the ATK-1 strain, which carries a Thr-to-Cys transition mutation, is transmitted efficiently between spouses, suggesting that miR-28 may play an important role in HTLV-1 transmission.

Human T-cell Leukemia virus, type 1 (HTLV-1)² infection is associated with a disease with poor prognosis known as adult T cell leukemia/lymphoma or HTLV-1-associated myelopathy (HAM/TSP) (1–5). Because the HTLV-1 virus is poorly infectious and has a very low antigenic variability, reducing the expression of viral antigens is critical in virus maintenance *in vivo*. Several studies suggest a tight dynamic between virus expression and immune control of proviral loads in HTLV-1-

infected patients (6, 7). The discovery of p30-mediated repression of HTLV-1 replication and its role in virus silencing suggests the existence of viral factors to help the virus persist in the host (8, 9). Other HTLV-1 proteins (Tax, basic leucine zipper (HBZ), p13, and p12) have also been reported to control virus expression (10–13).

In addition to viral factors, it is well established that the cellular environment has a profound impact on virus infection and replication. Many cellular genes can act as innate immunity factors to prevent replication and virus dissemination. In mammalian cells, viral infection is a potent trigger of the IFN response and activation of an antiviral state (14). Viruses have evolved multiple strategies to escape IFN (15). Recently, much attention has been focused on the role of non-coding RNA in virus pathogenesis. Virus-derived miRNAs can favor viral gene expression, virus replication, and virus infectivity (16) or even antagonize the IFN response (17). This mechanism has been described extensively for herpesviruses such as HSV1, Kaposi sarcoma-associated herpesvirus (KSHV), human cytomegalovirus (hCMV), and EBV (18–20) but appears to be absent in the HTLV-1 genome. In addition, some cellular miRNA may promote virus replication, as seen in the case of miR-122 and the hepatitis C virus (HCV) (21, 22), or a protective role, as reported for primate foamy virus, type 1 replication, which is inhibited by miR-32 (23). However, miRNA can also negatively regulate virus expression and infectivity. In fact, several anti-HIV cellular microRNAs (miR-28-5p, miR-150, miR-223, and miR-382) that target the HIV-1 genome have been reported to act in this way (24). These miRNAs are believed to participate in HIV latency because their level of expression correlates inversely with that of HIV-1 (25, 26).

To date, HTLV-1 has not been shown to encode or to be directly targeted by miRNA (27). HTLV-1 genome analyses revealed no conserved site for anti-HIV miRNAs miR-28-5p, miR-150, miR-223, and miR-382. However, we identified an octamer target site for miR-28-3p. The same miR-28 hairpin structure can be processed into mature products derived from each strand, termed miR-28-5p and miR-28-3p, which can then target different mRNAs. This study is the first to report the existence and mechanism used by a cellular

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² The abbreviations used are: HTLV-1, Human T cell leukemia virus, type 1; miRNA, microRNA; HIV-1, human immunodeficiency virus, type 1; AZT, zidovudine; HAM, HTLV-1-associated myelopathy; TSP, tropical spastic paraparesis.

miRNA to control HTLV-1 virus expression and to prevent virus transmission.

EXPERIMENTAL PROCEDURES

Cell Culture, Transfections, and Luciferase Assays—293T (ATCC) and BHK1E6 (28) cells were grown in DMEM with 10% FBS. Jurkat cells (ATCC) and the HTLV-1-transformed cell line MT-2 (16) were cultured in RPMI 1640 medium with 10% FBS. 293T, COS7 and BHK1E6 cells were transfected with Polyfect (Qiagen). Luciferase activities were assayed 48 h after transfection using the Dual-Luciferase reporter assay system (Promega). Peripheral blood mononuclear cells from healthy donors and HTLV-1-infected acute adult T-cell leukemia blood samples (29) were obtained after written informed consent and approved by consent in a study approved by the Institutional Review Board of the National Cancer Institute/National Institutes of Health.

Plasmids—Pri-miR-28 was amplified from healthy donor peripheral blood mononuclear cell DNA with the CTGGATC-CTGAAGGCAGGCCCTTCAAG (forward) and AAGAAT-TCCCATGTACCAGGCACAGAGA (reverse) primers and cloned into PCDNA3.1, pSIH-H1-copGFP, and pSIH-H1-puro vectors. The gag fragment from nucleotide 4836–5442 in HTLV-1 genomic RNA was amplified from the pBST primers CTCTAGAGTTTCTCGCCACTTTGATTTTATTC (forward) and AGAATTCTGGTATAGAGGGCTCGAGGATATG (reverse) and cloned into the pGL3-promoter vector. All mutants were made using the QuikChange® site-directed mutagenesis kit (Stratagene). To generate the miR-28-3pm mutant, only the miR-28-3p sequence was mutated, and miR-28-5p remained wild-type. All constructs were verified by sequencing. The HTLV-1 envelope-expressing vector, CMV-Env1-LTR, HTLV-LTR-luc, p-BST, and pc-Tax have been described previously (30–32).

Western Blot Analyses—293T cells were lysed in radioimmune precipitation assay buffer (50 mM Tris-Cl (pH 7.5), 150 mM NaCl, 1% Nonidet P-40, 1% sodium deoxycholate, and 0.1% SDS) containing a complete protease inhibitor mixture (Roche Diagnostics). Cell lysate concentrations were determined by a modified Bradford assay (Bio-Rad). Proteins were separated by SDS-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane. The membranes were probed with anti-HTLV-1p19 (catalog no. TP-7, ZeptoMetrix), anti-HTLV-1 p24, and anti- β -actin (catalog no. C-11, Santa Cruz Biotechnology). All secondary antibodies were from Santa Cruz Biotechnology.

Lentiviral Packaging and Infections—The vesicular stomatitis virus G glycoprotein (VSV-G) pseudotyped pSIH-H1-copGFP, pSIH-H1-copGFP-miR28, pSIH-H1-puro, and pSIH-H1-puro-miR28 viruses were generated as described previously (33). 293T cells were transfected with 20 μ g of lentiviral vectors, 10 μ g of pCMV-VSVG, and 10 μ g of pDNL6 using calcium phosphate (Invitrogen). Cell culture supernatants were collected at 48, 72, and 96 h after transfection. All viruses were concentrated by ultracentrifugation, resuspended in PBS, and frozen at -80°C until use. The viruses were filtered through a 0.45- μ m low-protein binding filter (Millipore) before infecting BHK1E6 or 293T cells. Jurkat cells were infected in the presence

of Polybrene and spinoculated at 1200 relative centrifugal force at room temperature for 1 h. Puromycin (Sigma) was added to 293T and Jurkat cells at 1 μ g/liter for selection 48 h after infection. The HTLV-1 envelope-pseudotyped pSIH-H1-copGFP virus was prepared as the VSV-G-pseudotyped virus except for replacement of pCMV-VSVG with the CMV-Env-LTR vector. Cell culture supernatants, including the virus, were collected 48 h after transfection, passed through a 0.45- μ m low-protein binding filter, and added to the 293T cell. 72 h later, 293T cells were fixed with 4% paraformaldehyde, and the number of fluorescent cells was counted by fluorescent microscopy, or the cells were resuspended and analyzed for GFP expression using FACS.

β -Galactosidase Reporter Assay—BHK1E6 cells (2×10^5) were cocultured with MT-2 cells (5×10^5) for 24 h. Then MT-2 cells were removed, and BHK1E6 cells were cultured for another 24 h. Monolayers were washed twice with PBS and fixed for 5 min with fixation buffer (1 \times PBS with 1% paraformaldehyde and 0.1% glutaraldehyde). The cells were washed twice with PBS and stained with a 200 μ g/ml X-gal (Sigma) solution. Cells were washed twice with PBS solution, and β -gal-expressing cells were counted twice by bright field microscopy. To inhibit the HTLV-1 reverse transcriptase, cells were treated with zidovudine (AZT, Calbiochem, 10 μ M) for 1 h at 37°C prior to coculture.

Circular Single HTLV-1 Detection Assay—The assay was done by coculture of BHK1E6 or Jurkat cells (2×10^5) with MT-2 cells (5×10^5). The cells were harvested 0–9 h after coculture for extrachromosomal DNA purification. Extrachromosomal DNA was extracted using the modified Hirt procedure (34). Briefly, the cells were washed twice with PBS and resuspended in 0.25 ml of buffer I (50 mM Tris (pH 7.5), 10 mM EDTA, 50 μ g/ml RNase A). Then 0.25 ml of buffer II (1.2% SDS) was added and incubated for 5 min. Then 0.35 ml of buffer III (3 M CsCl, 1 M potassium acetate, and 0.67 M acetic acid) was added and incubated for 10 min. After centrifugation at 13,000 rpm for 15 min, the supernatants were loaded onto miniprep columns (Qiagen). After centrifugation at 13,000 rpm for 1 min, the flow-through was discarded. The column was washed by adding 0.75 ml of washing buffer (60% ethanol, 10 mM Tris (pH 7.5), 50 μ M EDTA, and 80 mM potassium acetate) and centrifuged for 1 min. The flow-through was discarded and centrifuged for an additional 2 min to remove residual wash buffer. 100 μ l of elution buffer (1 mM Tris (pH 8.5) and 1 mM EDTA) was added and centrifuged for 2 min. 2 μ l of purified DNA was used as a PCR template for circular HTLV-1 detection or GAPDH. The primers used were GAAGAATACACCAACATCCCCATTCTCTAC pXF) and GGCGCTCGAATCCCGACGAG (pGagR). The GAPDH primers were the same as those used for real-time PCR. Prior to coculture with HTLV-1-producing cells, BHK1E6 and Jurkat cells were pretreated with AZT for 3 or 15 h, respectively. AZT was maintained in the culture medium during the coculture experiment.

RNA Extraction, RT-PCR, and Real-time PCR—Total RNA was extracted from cells using TRIzol (Invitrogen) and treated with DNase I to remove the DNA contamination. The reverse transcription was performed using high-capacity cDNA reverse transcription kits (Applied Biosystems) according to the instructions of the manufacturer. Real-time PCR was carried

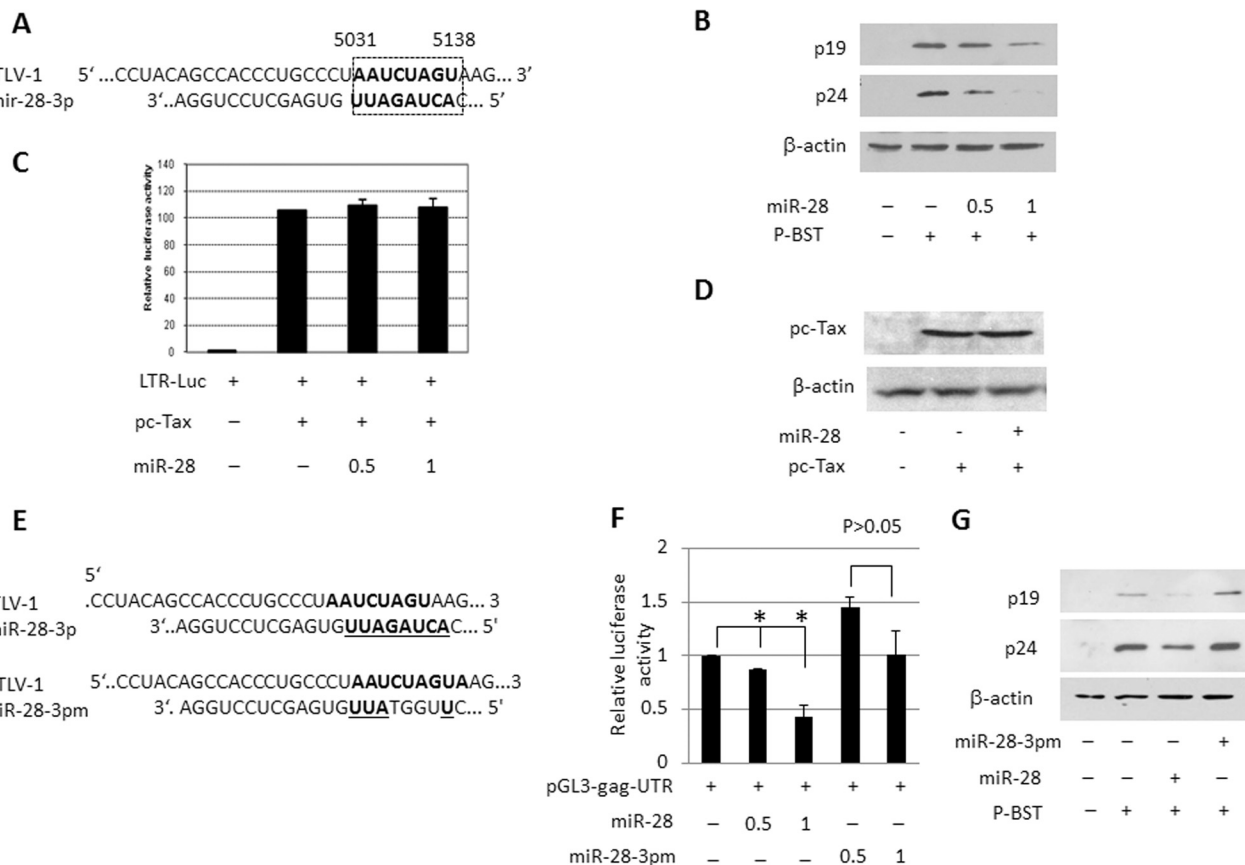


FIGURE 1. MiR-28-3p inhibits HTLV-1 replication and expression by targeting the HTLV-1 RNA genome. *A*, representation of the target sequence of miR-28-3p nucleotide 5031–5138 in the viral RNA. *B*, 293T cells were cotransfected with P-BST and increasing amounts of pcDNA3.1-miR-28. Equal amounts of transfected DNA were adjusted by adding the pcDNA 3.1 control vector to all samples. Extracts were made 48 h after transfection for Western blot analysis of the HTLV-1 gag products P19 and P24. *C*, 293T cells were cotransfected with HTLV-1 LTR-luc, pc-Tax, and increasing amounts of pcDNA3.1-miR-28. Equal amounts of transfected DNA were adjusted by adding the pcDNA 3.1 control vector to all samples. Extracts were assayed for luciferase (*Luc*) activity 48 h after transfection. *D*, 293T cells were cotransfected with 2 μ g of pc-Tax with or without 1 μ g of pcDNA3.1-miR-28. Equal amounts of transfected DNA were adjusted by adding the pcDNA 3.1 control vector to all samples. Extracts were made 48 h after transfection for Western blot analysis of Tax. *E*, the sequence of the miR-28-3p mutant (miR-28-3p pm), which was created in the miRNA seed sequences. *F*, cells were cotransfected with pGL3-gag-UTR-MT2, along with 0.5 and 1 μ g of pcDNA3.1-miR-28 or pcDNA3.1-miR-28-3p pm. Equal amounts of transfected DNA were adjusted by adding the pcDNA 3.1 control vector to all samples. Extracts were assayed for luciferase activity 48 h after transfection. Results are statistically significant (Student *t* test, $p = 0.0195$ and $p = 0.001$ for 0.5 and 1 μ g of transfected miR-28, respectively). *G*, 293T cells were cotransfected with P-BST, along with 1 μ g of pcDNA3.1-miR-28 or pcDNA3.1-miR-28-3p pm. Equal amounts of transfected DNA were adjusted by adding the pcDNA 3.1 control vector to all samples. Extracts were made 48 h after transfection for Western blot analysis of P19 and P24.

out using iTaqTM SYBR[®] Green Supermix (Bio-Rad) with the following sets of primers: GAPDH, GAAGGTGAAGGTGCGAGTC (forward) and GAAGATGGTGATGGGATTTC (reverse); LIM domain containing preferred translocation partner in lipoma (LPP), GTGCAATGTGTGTTCCAAGC (forward) and TGGCATAATAGGCTCCTTGC (reverse). Real-time PCR for mature miR-28-5p and miR-28-3p was performed with the miScript PCR system according to the instructions of the manufacturer. The miScript HiSpec buffer was used to prepare cDNA. The forward primers used for miR-28-5p and miR-28-3p were AAGGAGCTCACAGTCTATTGAG and CAC-TAGATTGTGAGCTCCTGGA, respectively. The expression of mature miR-28-5p and miR-28-3p was normalized to human RNU6B, which was provided in the kit. The expression of miR-28 in the stable cell lines was performed with pSIH-F and pri-miR-28-R primers.

RESULTS

Analyses revealed a conserved target site for miR-28-3p but not miR-28-5p in the proviral genome of HTLV-1 (Fig. 1A).

This binding site is located between nucleotides 5031 and 5038 of the HTLV-1 genomic mRNA, 196 bp after the stop codon of the polymerase gene. There is no conserved site for miR-28-3p in the genomes of HTLV-2 or HTLV-3. To confirm the functionality of the miR-28-3p site, we transiently transfected the HTLV-1 molecular clone pBST (35) in the absence or presence of a miR-28 expression plasmid. The results from these experiments demonstrated a dose-dependent decrease in HTLV-1 gag p19 and p24 products expressed from the full-length HTLV-1 genomic RNA (Fig. 1B). Because Tax controls the expression of viral genes by transactivating the viral LTR, we next wanted to demonstrate that a decrease in gag expression was not directly related to a miR-28-induced change in Tax expression. Because the miR-28-3p targeting site is absent from the viral *tax/rex* mRNA sequence (Fig. 1A), we used pc-Tax, an expression vector containing the *tax/rex* cDNA, as a control. We transfected cells with pc-Tax and the HTLV-1 LTR-luciferase construct in the presence or absence of miR-28. The results presented in Fig. 1C demonstrate that miR-28-3p has no effect on the

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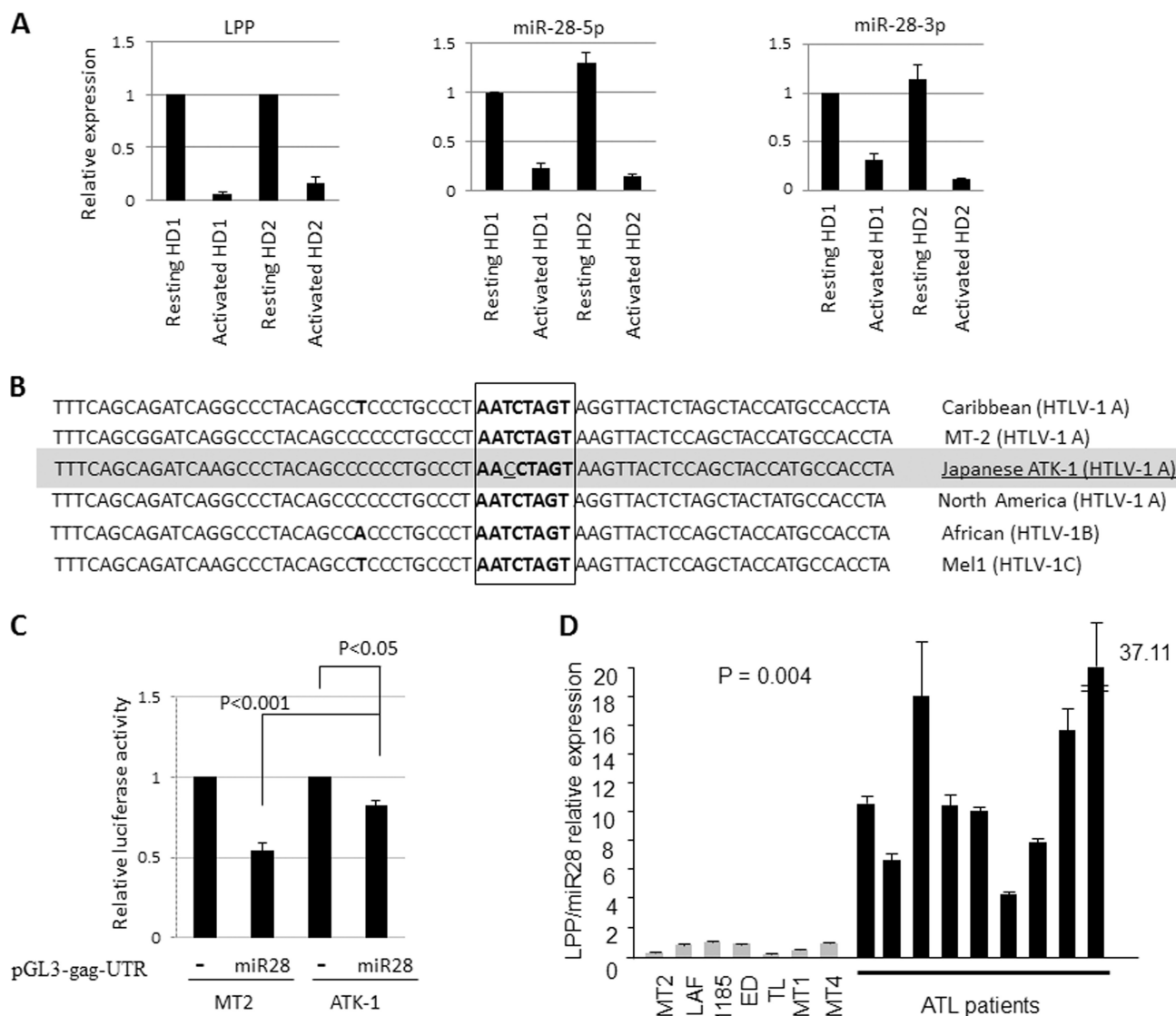


FIGURE 2. The Japanese ATK1 HTLV-1 1A subtype is more resistant to miR-28-3p inhibition. A, differential expression of mature miR-28-5p, miR-28-3p, and their host gene LPP in primary resting (after Ficoll without PHA and IL-2 stimulation) and activated peripheral blood mononuclear cells (stimulated with Phytohemagglutinin (PHA) and IL-2 for 48 h) in two healthy donors (HD1 and HD2), as detected by real-time RT-PCR. RNU6B expression was used as control for mature miR-28-5p and miR-28-3p and GAPDH expression for the LPP gene. B, the three major subtypes of HTLV-1 and their representative sequences around miR-28-3p target sequences. C, COS7 cells were cotransfected with pGL3-gag-UTR-MT2 or pGL3-gag-UTR-ATK-1, along with pCDNA3.1-miR-28. Extracts were assayed for luciferase activity 48 h after transfection. D, LPP gene expression was detected in HTLV-1 cell lines and freshly isolated ATL patient samples by real-time RT-PCR.

Tax cDNA sequence. In agreement with these data, Tax expression detected by Western blot analysis was not affected by the presence or absence of miR-28-3p (Fig. 1D).

To further confirm the specificity of miR-28-3p and its direct effect on the target sequence identified in the HTLV-1 provirus, we cloned a fragment of the HTLV-1 genome encompassing the miR-28-3p site. We cloned miR-28 and also created a mutated control sequence of miR-28-3p (Fig. 1E), and verified that this mutated miR-28-3p vector (miR-28-3 pm) does not have a target site in the HTLV-1 provirus. As expected, the luciferase reporter vector containing the miR-28-3p target fragment was affected in a dose-dependent manner by coexpression of miR-28-3p, whereas the miR-28 mutant (miR-28-3 pm) was not (Fig. 1F). The increase observed in the presence of a mutated miR-28-3p may be related to interference with endogenous wild-type miRNA28. Consistent with these data, the

transiently expressed HTLV-1 molecular clone was affected by miR-28-3p but not the miR-28-3p mutant, as shown by the reduction in both p19 and p24 gag only in the presence of wild-type miR-28-3p (Fig. 1G).

Real-time RT-PCR analyses of the LIM domain containing preferred translocation partner in lipoma (LPP), the locus encompassing the miR-28-3p and miR-28-5p sequences (36), revealed differential expression between activated and resting peripheral blood mononuclear cells (24). To further confirm these results, we directly measured the mature miR-28-5p and miR-28-3p in both resting and activated cells. Our results confirmed a 5- to 10-fold reduction of miR-28-3p expression in activated cells (Fig. 2A). It is well established that HTLV-1 virus particles can infect activated T cells but cannot infect resting T cells, raising the possibility that miR-28-3p acts as a restriction factor for HTLV-1 infection. We next investigated the natural

divergence of the miR-28-3p site in various HTLV-1 strains. The miR-28-3p target site was highly conserved in HTLV-1 subtypes B and C. However, the Japanese ATK-1 strain, subtype 1A, presented a natural polymorphism and Thr-to-Cys mutation within the miR-28-3p target site (Fig. 2B). The mutation is silent (AAT to AAC) and does not change the amino acid sequence in that region. Genetic studies of the transmission of the virus between spouses have shown that a virus carrying a Cys mutation is transmitted efficiently (37). We decided to test whether the Thr-to-Cys mutation affected the ability of miR-28-3p to suppress HTLV-1 virus replication. To this end, we introduced the Thr-to-Cys mutation in our gag-UTR reporter vector and transfected either the wild-type or mutated sequence in the absence or presence of miR-28. Importantly, the natural polymorphism occurring in the Japanese subtype 1A was more resistant to miR-28-3p inhibition (Fig. 2C). The transcriptional activity of the *LPP/miR-28* promoter is induced by constitutive activation of STAT5, which recruits transcriptionally active p53 to the *LPP/miR-28* promoter. Both active STAT5 and p53 are required for activation of the *LPP/miR-28* promoter (38). Although STAT5 is constitutively active in HTLV-1-transformed cells *in vitro* and ATL cells *in vivo* (39–41), studies have shown that p53 is generally inactive in HTLV-1-transformed cells *in vitro* (42). However, p53 function is reduced but functional in ATL cells *in vivo* (43). Consistent with these observations, we found that LPP (a surrogate marker of miR-28 expression) (44) was generally expressed at least 10-fold higher *in vivo* ATL samples compared with HTLV-1-transformed cells *in vitro* (Fig. 2D). Because the LPP gene is the host gene of miR-28-3p and their expressions are related (44), high LPP gene expression means high miR-28-3p expression. Interestingly, these data parallel virus expression, which is usually undetectable from ATL cells *in vivo* but abundant in transformed cells *in vitro*. These observations raise the possibility that miR-28-3p participates in silencing the virus *in vivo* to facilitate immune escape and virus persistence, and this warrants additional studies.

Because miR-28-3p targets the genomic viral RNA, we next hypothesized that miR-28-3p may restrict *de novo* infection by HTLV-1 virus particles. To test this hypothesis, we used a previously characterized reporter cell line stably transfected with an HTLV-1-LTR-Lac Z vector (28). Because the full-length HTLV-1 LTR is integrated in these cells, basal activity is extremely low, and only infected cells are revealed by a blue color after X-gal staining. We used this cell line to stably express pSI-H1-GFP or pSI-H1-miR-28-3p (Fig. 3, A and B). These cell lines, BHK1E6, BHK-GFP, and BHK-miR-28, were cocultured in the presence of an HTLV-1 virus-producing cell line, MT-2, for 1 day. After staining, the number of blue cells was quantified visually. Results from several independent experiments suggest that miR-28-3p-expressing cells were resistant to infection, as shown by a significant decrease in the number of blue cells in BHK-miR-28-3p cocultured with MT-2 (Fig. 3, C and D). We next demonstrated that the reduced Lac Z expression was not the result of a decreased ability of Tax to transactivate the integrated HTLV-1 LTR. Because we have shown previously that miR-28-3p is unable to target the Tax cDNA sequence (Fig. 1, C and D), we transfected the Tax expression vector. Results from

independent experiments indicated a similar number of blue cells in BHK, BHK-GFP, and BHK-miR-28-3p upon transfection of Tax (Fig. 3, E and F), suggesting that stable expression of miR-28-3p does not affect Tax expression or Tax ability to transactivate the HTLV-1 LTR or expression of Lac Z.

We next wanted to identify the infection step that is inhibited by miR-28-3p. In the absence of a cell-free infection system for HTLV-1, the effect of miR-28-3p on viral entry was tested using a lentiviral vector (pSI-H1-GFP) pseudotyped with either the HTLV-1 envelope (Env1) or the vesicular stomatitis virus envelope (VSV-G) as a control. The fact that miR-28-3p had no effect on VSV-G pseudotype particles (Fig. 4, C and D) suggests that miR-28-3p does not have a target site in the pSI-H1 vector sequence. Therefore, if miR-28-3p can affect HTLV-1 receptor expression or HTLV-1 viral entry, then we should see a difference in the number of infected cells. In fact, there was no significant difference in the efficiency of infection between control and miR-28-3p-expressing cells (Fig. 4, A and B). The VSV-G pseudotype was used as a control to confirm the specificity of our neutralization assays using HTLV-1 infected TSP/HAM patient serum to block infection by the Env-1 virus. As shown in Fig. 4, E and F, the HTLV-1 TSP/HAM serum effectively inhibited Env-1 pseudotype virus particle infection. As expected, the serum had no significant effect on particles pseudotyped with the VSV-G envelope (Fig. 4, G and H). Together, our results suggest that miR-28-3p does not affect viral entry but targets a post-entry step.

A major limitation in the HTLV-1 field is the absence of a reliable system to measure *de novo* infection. Despite a report of cell-free virion infection in dendritic cells (45), this system is difficult and relatively inefficient. HTLV-1 cell-free virus preparations are largely not infectious. HTLV-1 is mainly transmitted upon cell-cell contact (46, 47), and, as a result, it is difficult to discriminate between producing cells and newly infected cells. We developed a new sensitive assay for the detection and quantification of newly infected cells by HTLV-1. Specific primers were designed in the pX and gag regions so that only products of reverse transcription in newly infected cells could be amplified (Fig. 5A). Both single LTR circles and two-LTR circles are present at very low levels in MT-2 cells (Fig. 5A). Although two-LTR circles were also detected in infected cells, the frequency of these products was much lower than the single LTR circles, and, unlike single LTR circles, the relative amounts of two-LTR circles did not increase in infected cells (Fig. 5B). Therefore, the increase in single LTR circles (products of reverse transcription) can be used as a readout of newly infected cells. To demonstrate the specificity of our assay, we cocultured HTLV-1 virus-producing MT-2 cells with either BHK or Jurkat cells. Single-LTR circle DNA was not detected in any of these cell lines alone under the conditions described under "Experimental Procedures." Products of reverse transcription were easily detected following coculture with BHK or Jurkat cells (Fig. 5C). This was specific for newly infected cells because as treatment with the reverse transcriptase inhibitor AZT inhibited infection and the detection of single LTR products in cocultures (Fig. 5C). Importantly, our results demonstrate that the single-LTR circle detection method described here allows very sensitive and semiquantitative measurement of infection

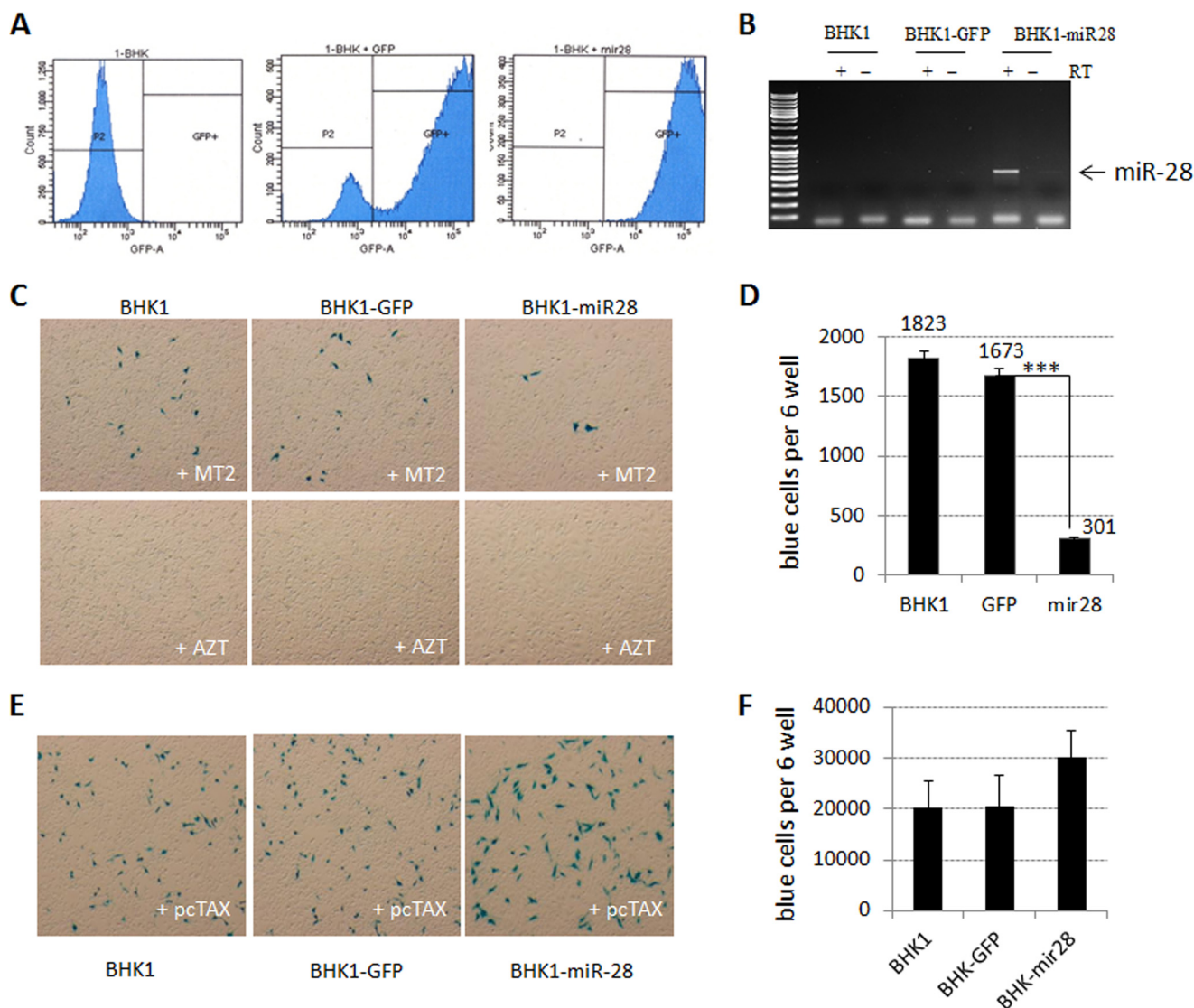


FIGURE 3. miR-28 can protect BHK1E6 cells from being infected by HTLV-1. A, BHK1E6 was infected by concentrated pSIH-H1-copGFP and pSIH-H1-copGFP-miR-28 virus. Then BHK1E6-pSIH-copGFP (*BHK1-GFP*) and BHK1E6-pSIH-copGFP-miR-28 (*BHK1-miR-28*) cells were analyzed with FACS for GFP expression. B, miR-28 expression in BHK1-miR-28 cells was analyzed by RT-PCR. C, BHK1E6, BHK1-GFP, and BHK1-miR-28 were infected by HTLV-1 through coculture with MT-2 cells for 24 h. 24 h after infection, cells were stained with X-gal to measure the infection. The images were taken at $\times 100$ magnification in a bright field. Representative images of the X-gal staining are shown. AZT ($10 \mu\text{M}$) was used as a control to prevent HTLV-1 infection. D, the average number of infected cells (blue cells) for BHK1E6, BHK1-GFP, and BHK1-miR-28 per 6-well plate. Mean \pm S.D. was calculated from two independent counts. The data are representative of three independent experiments. Results are statistically significant (Student's *t* test, $***, p = 0.015$). E, BHK1E6, BHK1-GFP, and BHK1-miR-28 cells were transfected with 0.1 μg of pc-Tax. The cells were fixed 2 days later and stained with X-gal. The images were taken at $\times 100$ magnification in a bright field. Representative images of the X-gal staining are shown. F, the number of blue cells for BHK1E6, BHK1-GFP, and BHK1-miR-28 that were transfected with pc-Tax. Mean \pm S.D. was calculated from two independent counts. The data are representative of two independent experiments.

in coculture systems. We next wanted to confirm the effect of miR-28-3p in both non-T cells and T cells. To this end, we generated a Jurkat cell line stably transfected with miR-28-3p (Fig. 5D). The presence of single LTR circles was monitored 0, 3, 6, and 9 h after initiation of coculture with HTLV-1-producing MT-2 cells. Infection of Jurkat cells, identified by reverse transcriptase product single-LTR circles, was detected as early as 3 h after contact with HTLV-1-producing cells (Fig. 5G), whereas infection of BHK cells was not seen until 6 h after contact with MT-2 cells (Fig. 5E). In coculture experiments, the amount of products of reverse transcription, detected by PCR and real-time PCR on cytoplasmic DNA, was reduced at 6 and 9 h in miR-28-3p-expressing cells compared with control cells (Fig. 5, E and F). Similarly, it is apparent that, in Jurkat cells, the

amount of single-LTR circle DNA at 3, 6, and 9 h is significantly lower when miR-28-3p is expressed (Fig. 5, G and H). Our results suggest that miR-28-3p inhibits a post-entry step, likely the HTLV-1 reverse transcription, thereby preventing the formation of the preintegration complex and leading to abortive infection.

DISCUSSION

In this study, we identified a novel mechanism for the control of HTLV-1 expression and the infection of target cells. The cellular microRNA miR-28-3p was found to target a sequence localized within the viral gag/pol genomic viral mRNA and reduce viral replication and gene expression in transiently transfected cells with an HTLV-1 molecular clone. All viral

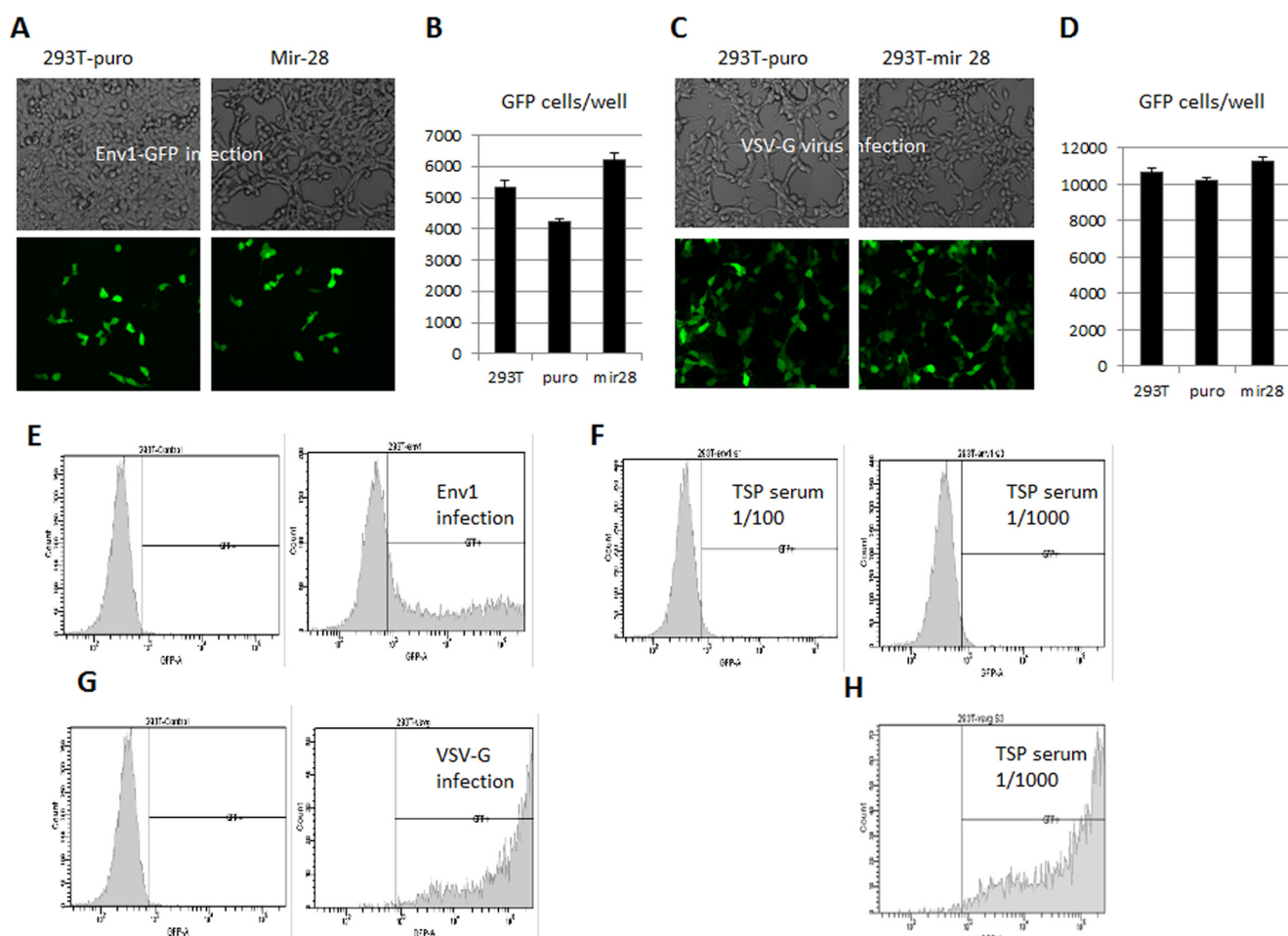


FIGURE 4. miR28 does not interfere with HTLV-1 envelope (*env1*)-mediated virus binding or entry. *A*, the same number of 293T, 293T-psih-h1-puro (293T-puro), and 293T-psih-h1-puro-miR-28 (293T-mir-28) cells was infected by HTLV-1 *env1*-pseudotyped viruses. Three days later, the cells were fixed. The images were made at $\times 200$ magnification for both bright field and GFP. *B*, the average number of GFP-positive cells for *env1*-mediated infection per 6-well plate. *C*, the same number of 293T, 293T-psih-h1-puro, and 293T-psih-h1-puro-miR-28 cells was infected by VSV-G-pseudotyped viruses. Three days later, the cells were fixed. The images were made at $\times 200$ magnification for both bright field and GFP. *D*, the average number of GFP-positive cells for VSV-G-mediated infection per 6-well plate. *E*, the HTLV-1 *env1*-pseudotyped virus was made by cotransfection of 293T cells with CMV-Env-LTR, pSIH-H1-copGFP, and PDLN6 plasmids. 293T cells were infected with the virus supernatants. GFP was analyzed three days after infection. *F*, serum from TSP/HAM patients can block the infection of HTLV-1 *env1*-pseudotyped viruses at both 1:100 and 1:1000 dilution. *G*, the VSV-G-pseudotyped virus was made by cotransfection of 293T cells with VSV-G, pSIH-H1-copGFP, and PDLN6 plasmids. 293T cells were infected with the virus supernatants. GFP was analyzed 3 days after infection. *H*, serum from TSP/HAM patients was diluted 1:100 and used in neutralization assays for HTLV-1 *env1*-pseudotyped viruses.

mRNAs that are derived from the genomic gag/pol RNA miR-28-3p can potentially reduce expression of all viral proteins. To demonstrate that reduced levels of p19 and p24 were not the result of a direct effect of miR-28-3p on Tax activation of the viral LTR, we performed luciferase assays and Western blot analyses using a Tax cDNA expression vector. The results from these experiments showed no effect of miR-28-3p on Tax-mediated transactivation or Tax expression, demonstrating that inhibition of HTLV-1 replication by miR-28-3p was independent of Tax.

HTLV-1 has a large tropism *in vitro* and *in vivo*. We next demonstrated that cells expressing miR-28-3p are refractory to HTLV-1 infection in cocultivation assays using both T cell and non-T cell target cells. Inhibition was not linked to receptor interaction and entry, as demonstrated by pseudotyping of the HTLV-1 provirus with a VSV envelope. In the absence of an efficient cell-free infection system, we developed a new technique for the detection of newly infected cells in a coculture

system. This assay is on the basis of the detection of reverse transcription intermediate single-LTR circles. Consistent with a previous report (48), our data show no increase in the presence of double-LTR circles following infection but, rather, a specific and significant increase in the presence of single-LTR circles as early as 2 h after mixing target and virus-producing cells (data not shown). The fact that miR-28-3p restricts HTLV-1 expression and infection is consistent with the high levels of miR-28-3p found in resting T cells and the inability of these cells to be infected by HTLV-1 without prior activation.

Interestingly, a natural feedback loop exists to control miR-28-3p expression in response to virus infection. Although *de novo* infection of target T cells activates the IFN antiviral response, miR-28-3p expression is increased significantly upon stimulation with IFN- α or IFN- γ (49). It is tempting to hypothesize that stimulation of miR-28-3p expression may, in turn, contribute to restrict virus expansion to neighboring cells by reducing virus expression. This may play a role in reducing local

miRNA-28 Blocks HTLV-1 Replication and Infection

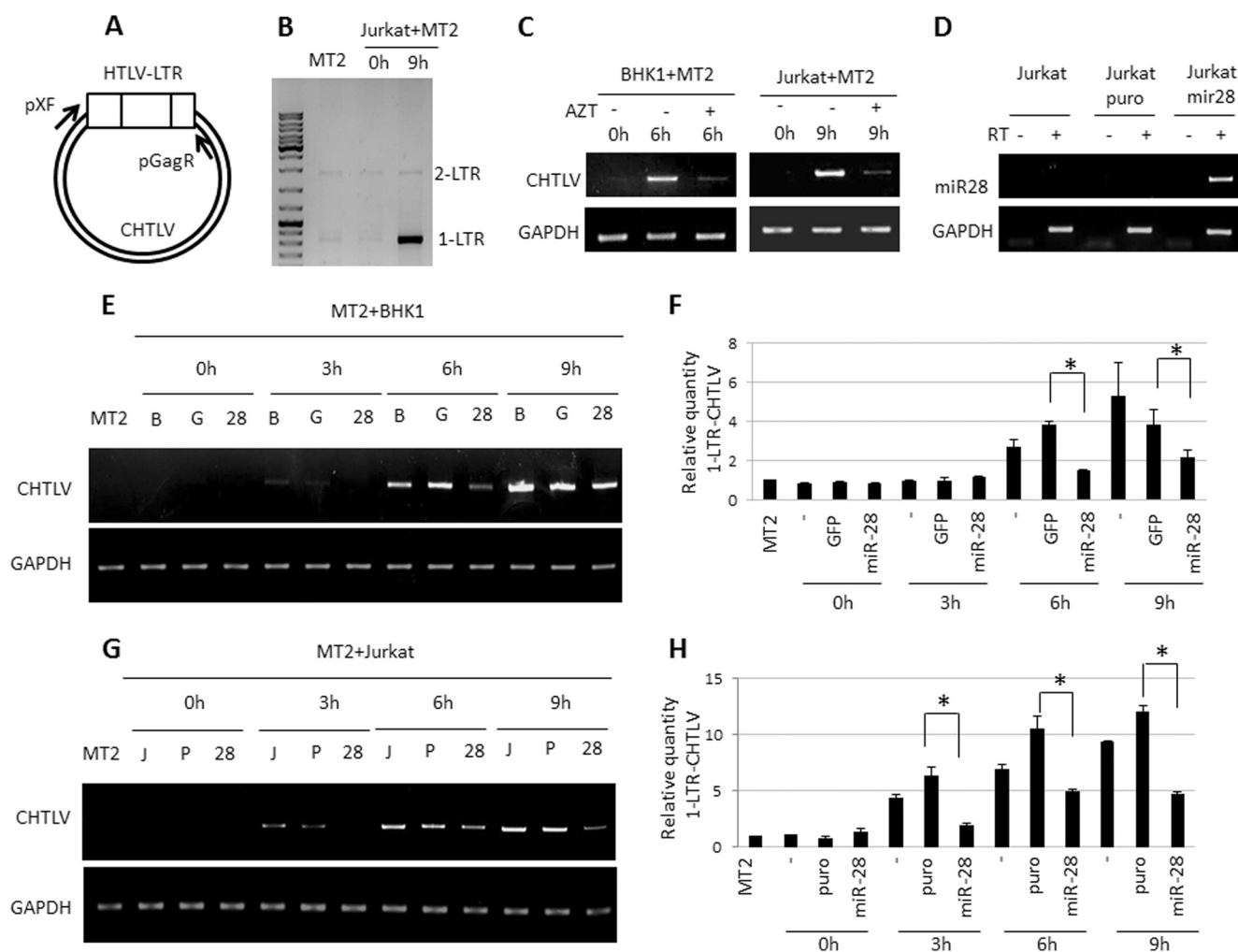


FIGURE 5. miR-28-3p can protect cells from the infection by interfering with the process of reverse transcription. A, schematic of the single-LTR circular HTLV-1 (1-LTR-CHTLV1) and the site of pX-forward (pX-F) and pGag-reverse (pGag-R) primers for the detection. B, Jurkat cells were cocultured with MT-2 for 0 and 9 h. Extrachromosomal cytoplasmic DNA was prepared from the cells and used as templates for PCR with the pX-F and pGag-R primer set. C, both BHK1 and Jurkat cells were cocultured with MT-2 cells for 6 or 9 h. AZT (10 μ M) was used as a control to block the infection. D, the expression of miR-28 was detected with RT-PCR. E, BHK1, BHK1-GFP, and BHK1-miR28 were cocultured with MT-2. Then 1-LTR-CHTLV1 was detected by PCR. F, semiquantitative detection of 1-LTR-CHTLV1 and 1-LTR-CHTLV1 was obtained by initial PCR amplification for 10 cycles with pX-F and pGag-R. Then 2 μ l of the PCR product was used as a template for real-time PCR with nested PCR primers, TCCGCGAAACAGAAGTCTGAA (forward) and TCCCGGAGGCTCTGAGCTTAT (reverse). *, $p < 0.05$. G and H, the same experiments as in E and F with Jurkat, Jurkat-pSIH-H1-puro (Jurkat-puro), and Jurkat-pSIH-H1-puro-miR28 (Jurkat-mir28).

inflammation and, possibly, the initial establishment of a latent reservoir.

Expression of antagomiRs directed against anti-HIV microRNAs (miR-28-5p, miR-125b, miR-150, miR-223, and miR-382) reactivated virus from latently infected T cells isolated from patients on suppressive, highly active antiretroviral therapy (50). Studies show that the miR-28-5p seed-matching sequence is the best conserved of all anti-HIV microRNAs, with 95% conserved of more than 5500 isolates (51). It is remarkable that miR-28 encodes two distinct miRNAs, miR-28-3p and miR-28-5p, targeting HTLV-1 and HIV-1, respectively. Other cellular microRNA regulating HIV-1 (miR-125b, miR-150, miR-223, and miR-382) had no conserved site within the HTLV-1 genome.

We found a natural polymorphism Thr-to-Cys mutation within the miR-28-3p target site in the Japanese ATK-1 viral genome strain, subtype 1A. Our studies demonstrate that the ATK-1 strain is relatively resistant to miR-28 expression, rais-

ing the possibility that this strain might be transmitted to resting T cells and dendritic cells more efficiently, and this warrants further studies. Importantly, the miR-28-3p target site is very well conserved in the HTLV-1 genome (90%). Target sequences identified by a Blast search, using as queries the miRNA-pairing sequences, revealed that, among 603 worldwide distributed sequences, 66 were identical to ATK1 in the miR-28-3p site. In Brazil, a new study showed that 95.5% are cosmopolitan transcontinental sub-subtypes and 4.5% are the ATK1 type (52).

Dynamic modulation of miR-28 expression is an attractive concept for HTLV-1 virus spreading because virus particles are able to transiently activate resting T cells, thereby reducing miR-28 expression and favoring infection. However, because IFN response is a strong inducer of miR-28 expression, the initial antiviral response may backfire, helping to conceal virus expression and to protect newly infected cells from being eliminated. Additional studies aimed at blocking miR-28-3p expres-

sion *in vivo* in an attempt to reactivate virus expression are warranted.

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